Cancer vaccines and immunotherapy

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Immunity and cancer

Peter C.L. Beverley, Miles W. Carroll and Peter L. Stern

Introduction

As early as the turn of the century, Paul Erhlich suggested that 'aberrant germs' (tumours) occurred at a high frequency in all humans but were kept in check by the immune system. Developments in understanding of the protective roles of antibodies and phagocytes in infectious disease in the early years of the century led to attempts to stimulate the immune system to reject tumours. The New York surgeon, Coley, used bacterial vaccines to cause a 'commotion in the blood' and occasional regressions following treatment or occurring spontaneously were taken as evidence of an effective immune response.

Early experimental work demonstrated that transplanted (allogeneic) tumours usually regressed. However, it was soon realized that this was a consequence of the genetic disparity of host and tumour and was revealing immune responses to foreign tissue transplants, not tumour antigens. However, what these early studies did show was that a strong immune response could prevent the growth of a tumour and cure the animal.

Immune surveillance

In the 1950s, Burnett[1] and Thomas[2] restated Erhlich's idea as the theory of 'immune surveillance'. It was proposed that the immune system was able to recognize abnormal cells, which were destroyed before they could develop into a tumour. Since tumours do develop in many individuals it was also suggested that the immune system played a role in delaying growth or causing regression of established tumours.

The strongest evidence for an effect of the immune system on tumours derives from the association between immunosuppression and increased tumour incidence. In kidney transplant recipients, many of whom have been followed for over 20 years, there is quite clearly a greatly increased frequency of tumours. On closer examination this data is not quite so straightforward as it at first appears. On the

Hepatitis B	Carcinoma of the liver
Human papillomaviruses (HPV) 16, 18 and other oncogenic types	Carcinoma of the cervix
Papillomaviruses	Carcinoma of the skin
Epstein–Barr virus (EBV)	Burkitt's lymphoma, nasopharyngeal carcinoma. Possibly Hodgkin's disease
Human herpes virus 8	Kaposi's sarcoma
Human T cell leukaemia virus-1 (HTLV-1)	Adult T cell leukaemia

Box 1.1. Viruses and human tumours

one hand, there is a large increase in the frequency of several tumours in which viruses are known to be involved (see Box 1.1); on the other, there is also a slight but definite increased risk for many other cancers in which viruses are not known to play a role[3].

These data strongly suggest that the immune response may be most effective in preventing the spread of potentially oncogenic viruses. Recent evidence that the incidence of hepatic carcinoma decreases following the institution of mass hepatitis B vaccination campaigns strongly supports the view that the immune system can be highly effective in preventing cancer, in this case by preventing infection with oncogene hepatitis B virus[4].

Figure 1.1

Experiments in immunosuppressed animals support the view that immune surveillance is largely directed towards viruses rather than tumours[5]. Many experiments have subsequently shown that cellular immune responses, mediated by thymus-derived (T) lymphocytes, are the key protective responses against viruses. These experimental data do not imply that there is *no* immune response to the majority of tumours but suggest that, for the majority of tumours, the immune response may be *relatively* ineffective (Figure 1.1).

The immune system and cancer

Although the evidence discussed above implies that the immune response against most nonviral tumours is ineffective, underlying the work discussed in the following chapters of this book is the assumption that antigen-specific immune responses against tumours *are* relevant. This assumption rests, first, on the idea that tumours are sufficiently distinct from other host cells that the immune response can distinguish between them; and, second, that an appropriate tumour-specific response

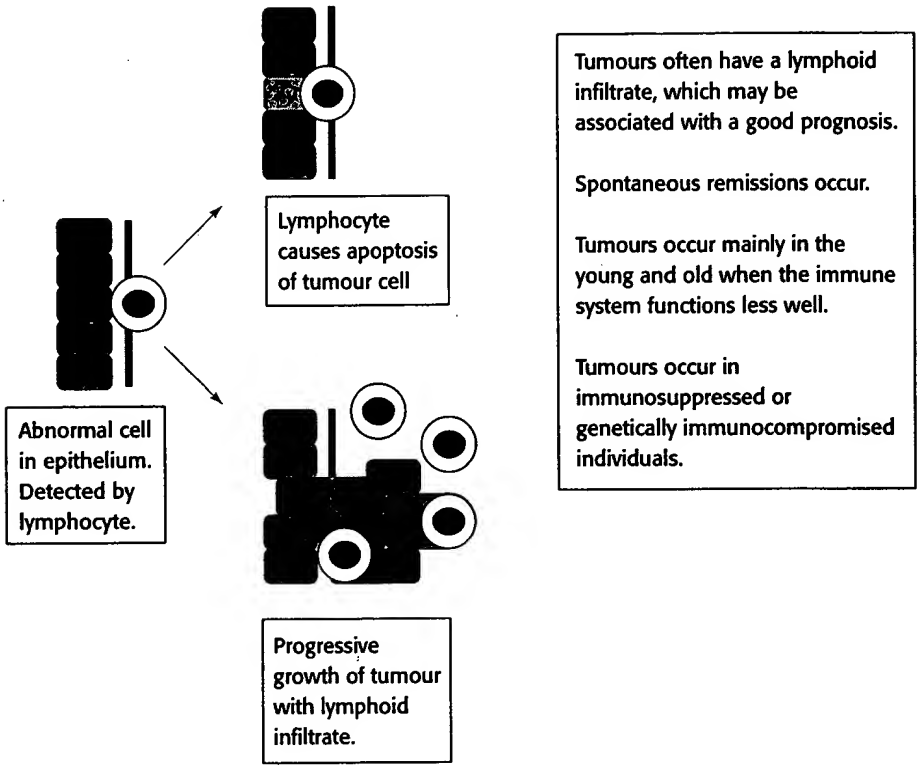

Figure 1.1 Immune surveillance and failure of surveillance

can cause tumour regression or elimination. A list of tumour antigen types and their potential immune recognition is given in Box 1.2.

Many tumour cells are distinguishable from corresponding normal cells using antibodies. First polyclonal antibodies then murine mAbs were used to identify tumour-associated antigens[6]. Not all aberrantly expressed molecules provoke an immune response by the host but passive immunotherapy may be directed at antigens which are well expressed on tumours, so long as side effects due to targeting of any normal cells expressing the antigen are acceptable. This principle underlies the use of most antibodies in immunotherapy and many trials have been carried out with mAbs which are known to target some normal cells as well as tumours[7].

There is also abundant evidence that nonviral tumours express antigens to which the host immune system can respond. Recently, host antibodies have been used to clone a number of antigens[8] (see Chapter 8) and pioneering work by Boon and his colleagues has firmly established that melanomas and other tumours express antigens recognized by T lymphocytes. They carried out in vitro mixed lymphocyte–tumour cultures to restimulate cytotoxic T lymphocyte precursors (CTLp).

Mechanism	Detection	Example
Point mutations, deletions and translocations generate new amino acid sequences	Host T cells	ras, p53, bcr-abl, etc.
Increased expression of highly tissue specific gene products	Host T cells	Mage-1, tyrosinase, prostate-specific antigen (PSA)
Expression of oncofetal antigens	Antibody	Carcinoembryonic antigen (CEA), α-fetoprotein
Aberrant glycosylation	Antibody Possibly host T cells	MUC-1, T and Tn antigens
Expression of normally inaccessible antigens	Antibody	CEA, α-fetoprotein
Viral antigens	Host T cells Antibody	HPV-16, EBV, HTLV-1 HHV-8, HepB
Expression of single cell specific antigens	Antibody Host T cells	Idiotypes of B- and T-cell tumours

Box 1.2. Origin and detection of tumour antigens

The resulting CTLs were used to define and clone many antigens of melanoma cells recognized by host T cells[9] (see Chapter 11).

That immune mechanisms can contain or eliminate tumours is also no longer in doubt. The data from animal experiments with allogeneic tumours showed that a tumour *could* be eliminated if completely foreign to the host. Later experiments showed that a small number of antigen-specific CTLs can cause complete regression of a tumour[10]. Similarly, in human posttransplant EBV lymphoma patients, infusion of immune T cells can cause complete tumour regression[11] and antibody-mediated therapy of a lymphoma caused regression of large tumour masses and a very long remission in the first patient treated[12]. The undoubted effects of IL-2 in some melanoma and renal cell carcinoma patients[13], and of BCG in bladder cancer (see Chapter 2), is also strongly suggestive of an effective cellular immune response even when induced nonspecifically.

Nevertheless, despite the undoubted existence of tumour-associated or tumour-specific antigens and the encouraging precedents for therapeutic effects described above, tumours do arise, grow and frequently kill patients. The remainder of this chapter attempts to illuminate this paradox by discussing the mechanisms of immune responses and how these might influence immunotherapeutic strategies.

Figure 1.

Antige

Antigen

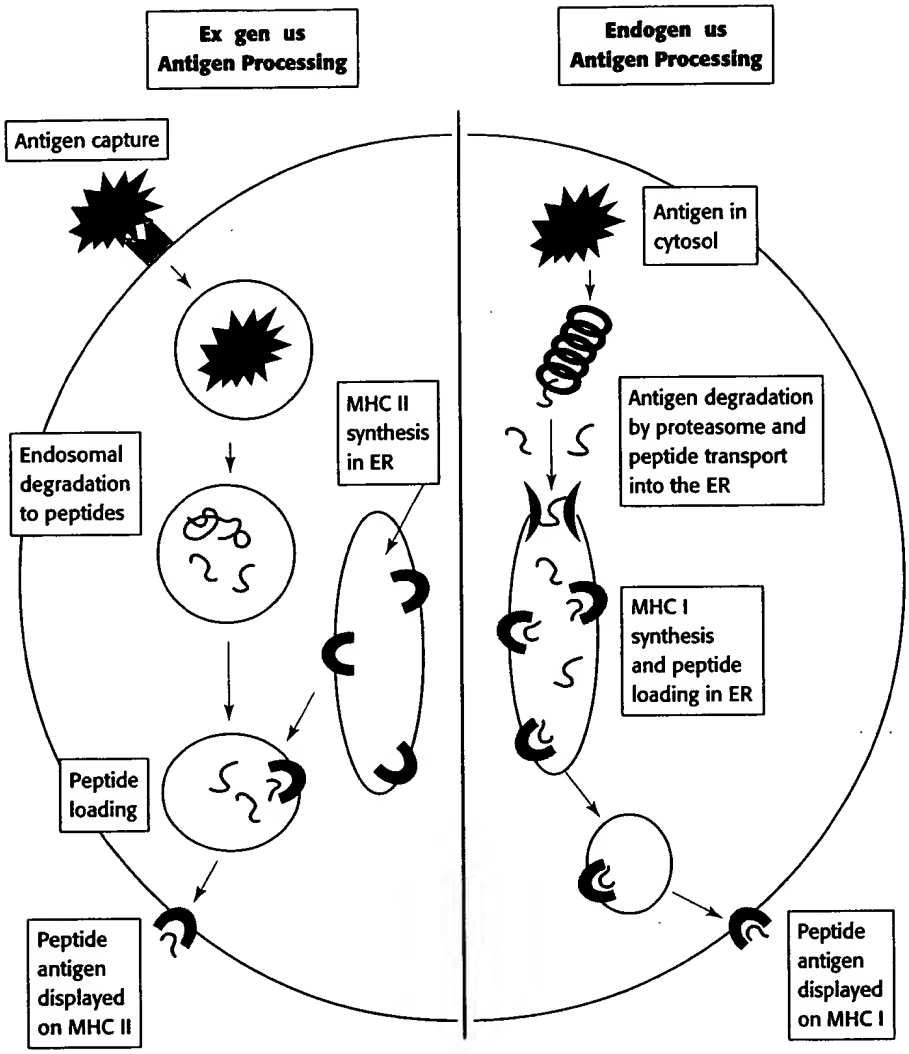

Figure 1.2 Antigen processing

Antigen recognition

Antigen processing

Antigen recognition by antibody occurs through the interaction of the binding site of an antibody molecule with a complementary three-dimensional structure (an epitope) on another molecule, the antigen. Although this may be complicated because the antigen is fixed in an array (for example in a cell surface) and because of the multivalent nature of antibodies, it is essentially a simple interaction. This is not the case for recognition of antigen by T cells. The key features of this process are illustrated in Figure 1.2.

Exogenous processing	Endogenous processing
The antigen must be taken up by specialized antigen-presenting cells. Danger signals are needed to initiate processing in APC.	Antigen must be synthesized in the cell. Processing can occur in any MHC-1 positive cell. Danger signals upregulate processing.
The peptides generated are dependent on: the specificity of processing enzymes, the glycosylation of the protein, the flanking sequences of the epitope, the cytokine microenvironment of the cell.	
	Peptides generated in the cytosol must be recognized by peptide transporters to enter the ER.
Peptides need to bind to the MHC I or II alleles of the processing cell in order to be displayed at the cell surface. Different alleles bind peptides with different motifs.	

Box 1.3. Factors influencing antigen presentation

Antigen processing for presentation on either major histocompatibility class I or II antigens (MHC I or II) is a complex process and the selection of peptides to be displayed is governed by factors which operate at each level of the processing mechanism (see Box 1.3). Processing of antigens is inefficient in the absence of 'danger signals'. These are nonantigen-specific signals, which indicate to the immune system that it has encountered a foreign material[14]; examples are bacterial lipopolysaccharide or specific sequence motifs of the DNA and RNA of micro-organisms. Danger signals are recognized by evolutionarily conserved receptors and are particularly effective in activating specialized antigen-presenting cells (APC) to process and present antigen.

The last step in the process, the binding of processed peptides to MHC molecules, is a critical step. The MHC is a highly polymorphic system and each allele binds a different set of peptides. For MHC class I the peptides are generally 8–10 amino acids long and binding is greatly influenced by one or two key 'anchor' residues, which fit into pockets in the MHC binding groove. The nature (charged, hydrophobic, etc.) and positions of the anchor residues in the peptide sequence make up a peptide binding motif which differs for each MHC allele. For MHC class II the peptides are generally 12–15 amino acids long but sequence motifs again influence peptide binding and the motifs may be allele specific. The consequence

of this specificity of binding is that not all new protein sequences may be recognized by T cells as foreign. For this to happen it is essential that some peptides generated from the new sequence by processing, bind with sufficient affinity to host MHC molecules to stabilize them and allow their transport to the cell surface[15,16].

As a rule, viral infection has been thought to be controlled by CTLs restricted through MHC I following endogenous processing of, for example, viral antigens. Recently it has become apparent that exogenous presentation is critical for induction of an immune response during viral infection of peripheral tissues[17]. This is perhaps not so surprising, since it would make little sense for the dendritic cells critical to the cross priming events necessary for subsequent CTL development, to be susceptible to various viral escape mechanisms apparent in other types of infected cells.

Self-tolerance

Since MHC molecules are unstable at the cell surface in the absence of bound peptides, the fact that most tissue cells express low levels of MHC class I molecules and a variety of APCs express MHC class I and II, implies that antigen processing proceeds in the absence of danger. Elution and sequencing of peptides from cells has shown that many of the peptides displayed are derived from normal self-proteins. Since, in general, the immune system does not respond to these self-molecules there must be mechanisms to prevent this.

Early experiments suggested that the thymus plays a key role in the development of T lymphocytes, including the selection of 'useful' T cells and the deletion of 'harmful' self-reactive cells[18]. Positive and negative selection are complex mechanisms but involve the interaction of the T cell receptor (TCR) of thymocytes with MHC–self-peptide complexes on APC. Depending on the affinity of this interaction and the presence or absence of other signals (co-stimuli), the developing thymocyte may survive and proliferate or die. In the bone marrow, similar selective mechanisms operate on developing B lymphocytes. Negative selection is not a foolproof mechanism so that autoreactive T and B cells exist in the periphery. In general, only B cells with relatively low affinity for self-antigens are present in peripheral lymphoid tissue. Development of high affinity antiself-reactive antibody requires somatic mutation in activated B cells, a process needing T cell help. Autoreactivity of B cells is therefore controlled by T cells.

After developing T cells leave the thymus to seed the periphery, the repertoire of available T cells continues to be shaped by a variety of mechanisms. These are either dependent on death or functional inactivation of self-reactive T cells by a variety of mechanisms, but these usually come into play when lymphocytes encounter antigen in the absence of adequate co-stimulation[19] (see Box 1.4).

Thymocytes with high affinity for self-MHC + self-peptide deleted by apoptosis.
Peripheral T cells encountering MHC + peptide in absence of co-stimuli may be deleted or anergized.
T cells may ignore antigens presented without co-stimuli.
Activation of T cells is dependent on concentration of MHC-peptide and amount of co-stimulation.
T-cell responses may be suppressed.

Box 1.4. Mechanisms influencing the peripheral T-cell repertoire

Co-stimulation and initiation of responses

T lymphocytes are the key regulators of the immune system. Activation of T cells to become effector cells, requires another signal (signal 2) in addition to that delivered through the TCR (signal 1). The nature of signal 2 has been the subject of intense investigation over the last few years and it has become clear that many different ligand–receptor pairs on the antigen-presenting cell and the T cell play a role (Figure 1.3). Some of these are listed in Box 1.5.

An important point in considering this cellular interaction is that it is a two-way process. As well as receiving signals from the APC, the T cell delivers signals to it and the consequence is activation and differentiation of both cell types. There is abundant evidence that the key antigen-presenting cell type in primary activation of T cells is the dendritic cell (DC)[20].

Recent evidence suggests that the sequence of events requires, first, that the DC is activated by 'danger' signals. Following this a process of maturation occurs, with up-regulation of key co-stimulatory molecules on the DC surface including CD80 and 86. In turn this initiates T cell activation and up-regulation of T cell surface molecules such as the IL-2 receptor, which is essential for growth of T cells. At the same time T cell CD154 (the ligand for CD40) is expressed and this delivers a very strong signal for further activation to dendritic cells. Very recently it has been demonstrated that ligation of DC–CD40 enables DC to acquire the ability to activate naive cytotoxic T cell precursors (CTLp) without the necessity for further signals delivered by T helper (Th) cells[21–23]. CD40–CD154 interaction is therefore a key stage in the DC–T cell interaction.

Cytokines produced by both cell types have effects on growth and differentiation of the cells. IL-12 and IL-4/10 have been shown to be particularly important in directing the production of Th-1 and Th-2 effector cells[24]. Chemokines control the migration of both DC and lymphocytes during the initiation of an immune response and its effector phase[25] (see Box 1.5).

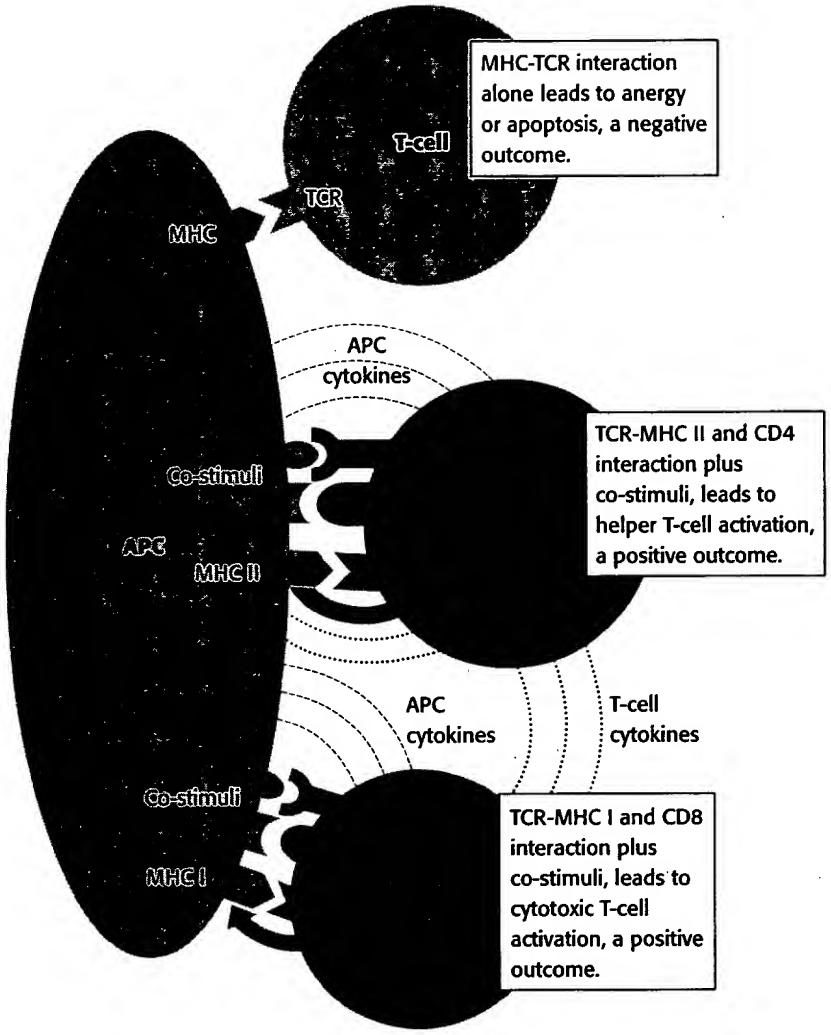

Figure 1.3 Co-stimulation

Priming of antitumour responses

In most immune responses to micro-organisms, priming is thought to occur in lymph nodes draining the site of infection. Initiation of the immune response requires a danger signal to alert the system. Without this DC will not be activated to process antigen, up-regulate co-stimulatory molecules and leave peripheral tissues to migrate to lymph nodes, where potentially responsive naive T cells encounter the antigen.

A small tumour may not initiate a response because it fails to deliver a danger signal. In contrast, once inflammation occurs in the tumour, perhaps through

APC	T cell	Outcome of interaction
MHC I	TCR	Delivery of Signal 1
MHC II	TCR	Delivery of Signal 1
CD4	MHC II	Facilitation of MHC II-TCR interaction
CD8	MHC I	Facilitation of MHC I-TCR interaction
CD56	CD2	Adhesion
CD11a/CD18	CD54	Adhesion and T-cell activation
CD80/86	CD28/CD152	Co-stimulation of T cells and activation of APC
CD40	CD154	Activation of DC and co-stimulation of T cells
	Cytokines in DC–T-cell interaction	
GM-CSFR	GM-CSF	DC growth and chemoattraction
TNFαR	TNFα	DC growth and maturation
IL-4R	IL-4	Bias to production of Th-2 effector cell sIL-6R
IL-10R	IL-10	Bias to production of Th-2 effector cells
IL-12	IL-12R	Bias to production of Th-1 effectors and CTL
IFNαR	IFNγ	Bias to production of Th-1 effectors and CTL
	Both DC and T cells express several chemokine receptors and chemokines	

Box 1.5. Cell-surface interactions between DC and T cells

breakdown of the epithelial barrier and entry of micro-organisms if the tumour is at a superficial epithelial site, or through tumour necrosis if it outgrows its blood supply, there will be an influx of inflammatory cells including DC. Necrotic or apoptotic tumour cells may provide a source of tumour antigen[26]. DC are stimulated and leave the tumour to migrate to draining nodes. Experimental evidence suggests that this is the main route for priming against tumour cells rather than direct priming by the tumour cells themselves[27]. Surprisingly this is the case for both the exogenous MHC class II and endogenous class I pathways, suggesting that in DC exogenous antigen can enter both processing routes[17].

While tumour antigen will eventually reach the draining node is there likely to be a high frequency of potential responder T cells? In theory, since many tumour antigens are unaltered self-molecules, high affinity responsive cells should have been deleted in the thymus, but in practice deletion is incomplete and T cells reactive to self-antigens including tumour-associated molecules, have been repeatedly demonstrated. Whether they are present at lower frequency or have lower affinity than T cells capable of responding to exogenous antigens, is currently unclear. In any case by the time patients present for immunotherapy, it is likely that tumour-

reactive cells will have been primed and that effector cells may have re-circulated to enter the tumour site. Studies of tumour-infiltrating lymphocytes (TILs) provide support for this concept[28]. Strategies for immunization against a growing tumour may therefore aim to prime naive lymphocytes or to boost pre-existing immunity.

Immunotherapeutic immunization strategies

The key factors in any attempt to generate or boost antitumour immunity is the delivery of relevant and immunogenic tumour antigens to professional antigen-presenting cells. This critical step is fundamental in the generation of any primary specific cellular or humoral immunity. This may result from a nonspecific activation induced locally by delivery of BCG (see Chapter 2) or the use of irradiated allogeneic tumour cells with cytokines and/or co-stimulatory molecules. Adjuvants (including cytokines) are usually utilized when immunizing with protein or peptides (Chapters 5, 6, 7 and 11) whereas pox viruses encoding tumour target antigens act as a potent inducer of the danger signals associated with APC activation etc. (see Chapters 3, 4 and 5). DNA vaccines must eventually lead to expression of tumour antigens and their processing by APCs (Chapter 12), whereas direct delivery of tumour antigens as proteins, by cell fusion or by cDNA to dendritic cells represents the most direct approach to attempt to generate antitumour immunity (see Chapter 13). These approaches are frequently biased by the prejudice that specific T cell immunity is likely to be of greater relevance in tumour therapy. However, the role of antibodies directly (e.g. Chapter 7) or indirectly (see Chapter 8) and generally in tumour immunity is probably being underestimated (see Chapter 10) and may be of critical importance in some virally associated tumours (Chapter 9).

One group of immunization strategies uses tumour cells as the immunogen on the assumption that many tumour antigens may not yet be defined. It is often assumed that the tumour cell presents its own antigens but since many tumours exhibit MHC class I down-regulation and lack MHC class II as well, they are unlikely to be optimal APC even if this does occur. However, many attempts have been made to remedy this by transduction of the tumour cells with genes for some of the missing molecules. The logic of this is obscure if immunization occurs, not through presentation of antigens by tumour cells themselves, but by processing of tumour-derived antigen in host antigen presenting cells. Additionally, although one or two co-stimulatory or MHC molecules can be inserted into a tumour cell, it is highly unlikely that this will make it present antigen as efficiently as a 'professional' antigen-presenting cell (usually a dendritic cell).

An alternative type of strategy attempts to ensure that tumour antigen reaches antigen-presenting cells. This can be achieved by transducing tumour cells in vivo with genes for cytokines or chemokines (e.g. GM-CSF), which might attract

Aim	Strategy
Prime naïve T cells	Present tumour antigen with adjuvant (danger signal) to initiate a response and clonal expansion
Boost memory T cells	Present tumour antigen with adjuvant to induce activation and further clonal expansion
Activate pre-existing specific effector cells	Local or systemic cytokines (IL-2)
Activate nonspecific effector mechanisms	Local or systemic cytokines
Relieve immunosuppression or modulate the immune response	Local or systemic cytokines, immunomodulatory agents

Box 1.6. Active immunotherapy

antigen-presenting cells to the lesion[29]. Alternatively, in vitro grown tumour cells may be transduced, inactivated and used as an immunogen. A logical extension is to use DC directly loaded with tumour antigens in vitro as the immunogen[26]. There are now many approaches focused on specific or nonspecific immunization using DC (see Chapter 13).

The antigen need not be in the form of tumour cells since tumour antigens are rapidly being defined. Subunit vaccines of various types have the advantage that they remove irrelevant molecules and potentially interfering or immunosuppressive ones. The down-side is that if few T cell epitopes are included in the vaccine there may be no epitopes, which bind with high affinity to the MHC alleles of some vaccinees, since each allele binds epitopes with a particular sequence motif[30]. Various strategies are summarized in Box 1.7.

Strategies aimed at DC have the advantage that ultimately they target lymph nodes, mimicking the physiology of a normal immune response (Figure 1.4). Another possible advantage of methods employing in vitro transduced cells is that they may be injected at a site distant from the tumour, avoiding the problem that the tumour itself may produce immunosuppressive substances such as the cytokine TGFβ[31] and that these may reach tumour-draining nodes. Unfortunately, tumour patients' T cells sometimes exhibit poor responsiveness in vitro, suggesting a systemic immunosuppressive effect of tumours[32]. This has been attributed to abnormalities in expression of the CD3ζ chain, which is involved in signal transduction. What causes this is a matter of debate and how specifically related to cancer is the defect is not established, but the functional abnormality can sometimes be reversed in vitro and possibly in vivo by IL-2[33]. This is discussed in Chapter 5.

Effect

Problems

The tumour antigens need to be defined.
Single T-cell epitopes are often MHC allele specific.
Subunits lack danger signals.

Advantages/solutions

Removal of irrelevant but competing or suppressive antigens.
Adjuvants, helper antigens, cytokines or co-stimuli can be easily combined with subunits.
Multiple epitopes from different antigens can be combined in epitope strings to overcome the allele problem.
The vaccine can be designed to generate appropriate immune responses.
Vectors can be tailored to achieve optimal immunization.

Methods for administration

Peptides with or without adjuvant.
Recombinant proteins with or without adjuvant.
Glycoconjugates with helper epitope and adjuvant.
Recombinant viruses (e.g. vaccinia-MUC-1 or Vac-HPV-16 E6 and E7, with or without cytokines).
DNA, combining antigen with co-stimuli or cytokines.

Box 1.7. Subunit vaccine strategies for tumours

Effector function

The immune system has multiple effector mechanisms for combating invading micro-organisms (see Box 1.8) but it remains unclear which of these are most effective against tumours. Complicating the issue is the enormous variation in the behaviour of different tumour types, so the most important effector mechanism may well differ depending on the tumour type.

The available animal experimental data is not particularly helpful. Evidence for some mechanisms is mainly based on in vitro experiments and extrapolation from immunohistology. For example, macrophages are abundant in many tumours and can be shown in vitro to inhibit the growth of tumour cells, but it is less clear what role they play in vivo. The role of antibodies produced by the host itself is also controversial, although such antibodies have proved to be an important tool for definition of tumour-associated molecules (Chapter 10). On the other hand, monoclonal antibodies (mAbs) have been shown in humans to be able to localize tumours and have been demonstrated convincingly to delay the onset of tumour progression and increase survival in a randomized trial of a mAb as adjuvant

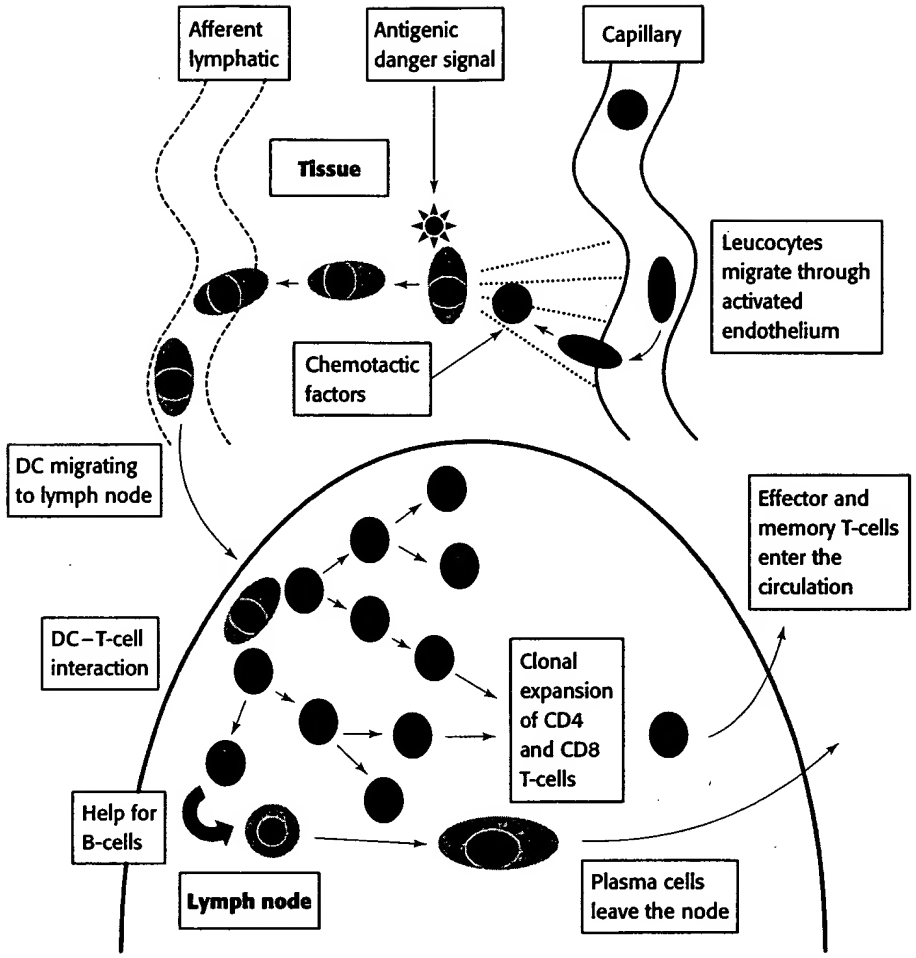

Figure 1.4 The anatomy of an immune response

therapy for colon cancer[7]. The mechanism of this effect has not been elucidated, nor are the mechanisms which have occasionally led to remissions in lymphoma patients treated with anti-idiotypes or to tumour dormancy in experiments in a murine model[34] (see Chapter 8). As yet there have been few attempts to generate high titre antibodies to tumour-associated antigens in humans except in trials targeted to idiotypes of B cell tumours[35] (see Chapter 12). Whether antibodies to other surface antigens, generated by active immunization of the host, might be effective particularly against small metastases, remains to be properly investigated.

The evidence that allograft rejection is mediated by T cells has led many investigators to focus on T lymphocytes as antitumour effectors. In mouse experimental models, there is evidence for the participation of T cells in protection against

Humoral

Antibody blocking (for example, of growth factor receptors).
Antibody-induced apoptosis.
Antibody- and complement-mediated lysis.
Cytokine-mediated cytostasis or cytotoxicity (e.g. cytostatic effects or interferons or cytotoxicity of TNFα).

Humoral and cellular

IgE-mediated allergic reactions involving basophils and mast cells.
Antibody-mediated cellular cytotoxicity by natural killer cells and macrophages.

Cellular

Natural killer cell cytotoxicity.
Cytostasis and cytotoxicity mediated by activated macrophages.
T cell cytotoxicity
  by $\alpha\beta$ T cells
  by $\gamma\delta$ T cells

Box 1.8. Immune effector mechanisms

tumour challenge (an artificial situation in which the animal is first immunized against the tumour and then challenged with viable tumour cells) and in rejection of established tumours. Evidence described earlier indicates that small numbers of activated cytotoxic T lymphocytes (CTL) can certainly eliminate relatively large tumour masses under optimal circumstances, and many human tumour antigens have been defined using CTL, so that there continues to be a concentration of effort on immunization against MHC class I binding epitopes.

The overwhelming problem of this strategy is the loss of MHC class I molecules, which is such a prominent feature of human tumours. This may be allele specific or global and several molecular mechanisms have been defined, including mutations in the peptide transporters, in MHC molecules themselves and in β2-microglobulin[35]. Loss of MHC molecules suggests that the T cell immune response applies selective pressure to tumour cell populations, but it also implies that by the time a tumour is detectable it may already have been selected for resistance to the T cell antitumour immune response. Although natural killer (NK) cells may recognize better the cells which express low levels of MHC[36], few NK cells can be demonstrated in most tumours and infusions of lymphokine activated killer (LAK) cells have not been notably successful. All this suggests that MHC loss is likely to be a major bar to immunotherapy aimed at stimulating CTL.

C nclusi ns

Immunotherapy has undergone many ups and downs during this century. What is unarguable is that the immune system can destroy large tissue masses if it can be brought to bear on them. Recent data suggest that human tumours do differ from their hosts sufficiently for them to be recognized as foreign, although the frequency and affinity of the responding cells is not clear. Tumours may be initially poor immunogens because they lack danger signals and produce immunosuppressive substances, which interfere with immune responses. Once an immune response is generated, there is evidence for escape through down-regulation of MHC molecules.

All this makes it clear that therapeutic active immunization may be difficult. Early institution of immunotherapy is likely to be more effective, when the tumour has had less chance to escape and the immune system has not been damaged by chemotherapy. It also makes sense to target as many antigens as possible, making escape more difficult. Rapid progress in definition of tumour antigens and improvements in methods for immunization, will mean that it will at least be possible to test whether optimal immunization to obtain a large and broadly targeted response, will be an effective therapeutic anticancer modality. This volume details the present state of the art, although as yet this goal has not been reached.

Historically, immunization has been most effective when administered prophylactically. Definition of more and more tumour antigens may open the way to prophylactic immunization for nonviral as well as viral tumours, at least in high-risk groups. The problem of immunoselection may in the future be overcome by using T cells engineered to recognize antigen through an introduced antibody receptor. Antibody itself can be effective. A conservative view is therefore that, in the next decade, some forms of immunotherapy will take their place as standard cancer treatment.

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10

Serologically identified tumour antigens as cancer vaccines

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Introduction

Vaccination strategies for the treatment of human cancer depend on the existence of tumour antigens which are able to elicit specific immune responses in the tumour-bearing host. The specific recognition of antigens by the immune system is accomplished by two targeting systems: CD4+ and CD8+ T lymphocytes recognize processed antigens presented on MHC class II and class I molecules, respectively, while B lymphocytes produce antibody molecules that bind specifically to unprocessed antigens. The analysis of humoral and cellular immune responses in cancer patients had indicated for a long time that cancer-specific antigens do indeed exist and are recognized by the immune system of the tumour-bearing host[1]. However, the molecular nature of these antigens remained unclear until cloning techniques were developed that used established cytotoxic T lymphocyte (CTL) clones[2] or circulating antibodies[3] as probes for screening of tumour-derived expression libraries. The CTL approach and the antigens identified by it are reviewed elsewhere in this book (Chapter 11). This chapter is intended to give an introduction to the serological approach, to summarize the current status of antigens identified and to provide a perspective for the use of these antigens for cancer immunotherapy.

Rationale for using the antibody repertoire of cancer patients for the identification of tumour antigens

A variety of in vitro studies and animal tumour models demonstrated that CTLs are the protagonists of an effective cytotoxic antitumoural immune response and motivated the search for antigens recognized by CD8+ T lymphocytes. The necessity of established precharacterized CTL clones with tumour-cell restricted reactivity is the major obstacle of the CTL-based cloning approach and was the main reason why the majority of antigens defined hitherto were identified in malignant melanoma[4]. Even though tumour immunology has been CTL-centric in the last decade, it is common knowledge that antitumour immune recognition is a

concerted action. A large body of evidence points to a coordinated recruitment of CD4+, CD8+ and B-cell responses to the same tumour antigen, and suggests that once immune recognition of an antigen is elicited, it is not restricted to merely one effector system. Furthermore, it is frequently argued that the CTL repertoire of cancer patients is deleted for many relevant CTL precursors. However, it is quite unlikely that a concomitant antibody response towards antigens (in particular intracellular ones) for which respective CTLs have been deleted, would also be erased[5]. Thus, specific antibodies may be the persisting hallmark of a substantial tumour–immune system confrontation and may help to trace back to deleted CTL specificities. Based on these rationales, we designed a novel strategy using the antibody repertoire of cancer patients for the molecular definition of antigens. Once identified serologically, these tumour antigens were analysed subsequently for T-lymphocyte-recognized epitopes presented by MHC class I or II molecules.

The SEREX approach

For the systematic and unbiased cloning of tumour antigens recognized by the antibody repertoire of cancer patients, we developed a serological cloning approach, termed the SEREX (serological analysis of tumour antigens by recombinant cDNA expression cloning) approach[6,7]. For SEREX, cDNA expression libraries are constructed from fresh tumour specimens, packaged into lambda-phage vectors and expressed recombinantly in *Escherichia coli*. Recombinant proteins expressed during the lytic infection of bacteria are transferred onto nitrocellulose membranes. These are incubated with diluted and extensively preabsorbed autologous patient serum. Clones reactive with high-titred IgG antibodies are visualized using an enzyme-conjugated secondary antibody specific for human IgG. Positive clones are subcloned to monoclonality, thus allowing the direct molecular characterization. The SEREX approach is technically characterized by several features:

(1) There is no need for established tumour cell lines and precharacterized CTL clones.

(2) The use of fresh tumour specimens restricts the analysis to genes that are expressed by the tumour cells in vivo and circumvents in vitro artefacts associated with short- and long-term tumour cell culture.

(3) The use of the polyclonal (polyspecific) patient's serum as a probe for immunoscreening allow for the identification of multiple antigens with one screening course.

(4) The screening is restricted to clones against which the patient's immune system has raised high-titred IgG and/or IgA antibody responses indicating the presence of a concomitant T-helper lymphocyte response in vivo.

(5) As both the expressed antigenic protein and the coding cDNA are present in the same plaque of the phage immunoscreening assay, identified antigens can

be sequenced immediately. Sequence information and excised cDNA inserts can be directly used to determine the expression spectrum of identified transcripts by Northern blot and reverse transcription polymerase chain reaction (RT-PCR).

(6) The release of periplasmic proteins involved in protein folding during phage-induced bacterial lysis allows at least partial folding of recombinant proteins and provides the basis for the identification of linear as well as nonlinear epitopes. This has been confirmed by the expression of transcripts which code for enzymatically active proteins (our unpublished results). In contrast, epitopes derived from eucaryotic posttranslational modification (e.g. glycosylation) are not detected by the phage immunoscreening assay.

Antigens identified by SEREX

As stated above, the SEREX approach allows for the simultaneous identification of multiple antigens using the antibody repertoire of a single cancer patient. The analysis of a variety of neoplasms demonstrated that all hitherto investigated neoplasms are immunogenic in the tumour-bearing host and that immunogenicity is conferred by multiple antigens. As the antitumour antibody repertoires from individual cancer patients vary considerably, a large number of antigens could be identified by SEREX. The proliferation of the technology to, and the cooperation with, many other laboratories in the coordinated analysis of different types of human cancers will provide the systematic typing of the expressed immunogenic human cancer genome. For the systematic documentation and archivation of sequence data and immunological characteristics of identified antigens, an electronic SEREX database was initiated by Dr Lloyd Old and implemented by Victor Jongeneel and colleagues of the Ludwig Cancer Research Institute. It provides a variety of means for sequence analysis and homology searches.

Classification of tumour antigens

By December 1998 more than 900 entries have been made in the SEREX database, the majority of them representing independent antigens. These include known tumour antigens such as the melanoma antigens MAGE-1, MAGE-4a and tyrosinase, which demonstrates that at least some of the serologically identified antigens are also targets for CTL. A second group of antigens is comprised of transcripts that are either identical or highly homologous to known genes which have not been known to elicit immune responses in humans, e.g. kinectin, a microtubule-associated transporter of golgi vesicles. The third group of serologically defined antigens consists of previously unknown genes, such as HOM-HD-21, a new galectin from a tissue affected by Hodgkin's disease[8]. The abundance of antigens and the fact that

Table 10.1. *Categories of tumour-associated antigens identified by SEREX*

Class	Antigen	Homology/identity	Source
Cancer testis antigens	HOM-MEL-40	SSX-2	Melanoma
Differentiation antigens	HOM-MEL-55	Tyrosinase	Melanoma
Overexpressed gene products	HOM-HD-21	Galectin-9	Hodgkin
Mutated gene products	NY-COL-2	p53	Colon cancer
Splice variants	HOM-HD-397	Restin	Hodgkin
Gene amplification products	HOM-NSCLC-11	eIF-4g	Lung cancer
Cancer-related autoantigens	HOM-MEL-2.4	CEBPgamma	Melanoma

a large portion of them is encoded by previously unknown genes, calls for a precise procedure to assess the role of the identified transcripts and observed immune reactions in the course of the malignant disease. This is performed by a three-step analysis which comprises a sequence analysis (search for tumour-associated sequence alterations) with subsequent homology search, expression studies in neoplastic and normal tissues and the determination of the immunogenic spectrum as assessed by the frequency of antibodies in sera from cancer patients and healthy controls. Based on the results of this basic analysis the SEREX antigens can be assigned to different groups (Table 10.1).

Expression studies are performed by Northern blot analysis using labelled cDNA as probes and by RT-PCR with transcript-specific primers. These investigations identify transcripts with a tumour-associated expression. Tumour antigens with high or frequent expression in tumour cells or tissues are restricted (i.e. no or very low expression) in normal tissues and are of special interest for cancer immunotherapy. Antigens with such a type of expression fall into several expression categories.

Cancer testis antigens (CTA) are selectively expressed in a variety of neoplasms (in a lineage-independent manner), but not in normal tissues except for testis. Examples are members of the MAGE gene family, which had already been defined by CTL approaches, and several new antigens such as HOM-MEL-40 and NY-ESO-1 which will be discussed in more detail below.

Differentiation antigens demonstrate a lineage-specific expression in tumours, but also in normal cells of the same origin; examples are tyrosinase and GFAP (glial fibrillary acidic protein) which are antigenic in malignant melanoma and glioma, but are also expressed in melanocytes or brain cells, respectively.

Overexpressed genes code for many tumour antigens identified by SEREX. The members of this class are expressed in low levels in normal tissues (usually detectable by RT-PCR), but are up to 100-fold overexpressed in tumours. An example is

HOM-RCC-3.1.3, a new carbonic anhydrase which is overexpressed in a fraction of renal cell cancers[9]. The overexpression of a transcript may result from gene amplification as demonstrated for the translation initiation factor eIF-4g in a squamous cell lung cancer[10].

Antigens encoded by mutated genes have been demonstrated only rarely by the serological approach, with mutated p53 being one example[11].

Tumour-specific splice variants of otherwise ubiquitously expressed genes can also result in tumour-associated immunogenicity. Similar to CTA, these splice variants can display an expression pattern that is restricted to tumours and testis (unpublished data).

Virus-encoded antigens that elicit an autologous antibody response have also been detected by SEREX, e.g. the env protein of the human endogenous retrovirus HERV-K10[12] which was found in a renal cell cancer.

Cancer-related autoantigens elicit antibody responses in patients with different types of cancer, but not in individuals without malignant diseases. Examples for this class are p53[13] and HOM-TS-64/kinectin (unpublished data).

Cancer-independent autoantigens elicit autoimmunity that is not related to neoplastic disease. An example is HOM-MEL-23 which is identical to the proliferating cell nuclear antigen (PCNA), a known nuclear autoantigen.

The designation of an antigen to a particular category validate a newly identified molecule for different fields of cancer research. The identification of a multitude of new genes (including mutated products) with tumour-associated expression provides new information and new targets for a better understanding of cancer biology. The transcripts derived from these genes and the proteins encoded by them as well as the associated immune responses may be clinically useful as novel diagnostic or prognostic cancer markers. Ongoing studies with large groups of patients will show which subpopulations of tumour patients develop antibodies to particular antigens and whether these immune reactions may be useful as early markers for the serodiagnosis of cancer.

Optimization of cancer vaccines

From the therapeutic point of view, the main application for cancer antigens is cancer vaccination. For this purpose it is important to choose the right targets. For clinical as well as for technical and economical considerations, it is apparent that only a few antigens can be chosen as targets for controlled clinical cancer immunotherapy studies. It will therefore be important to define rationales for the selection of suitable antigens. In this regard a number of requirements for the rational selection of vaccine candidates can be listed. The main idea of cancer vaccination is to induce an effective specific cytolytic immune activity against tumour cells. To avoid

major side-effects by destruction of nonneoplastic cells the molecular targets of the induced cytolytic activity should not be expressed or at least not recognized on tissues which are essential for the health of the vaccinated individual. With respect to specificity several classes including CTA, differentiation antigens, tumour-associated overexpressed gene products, mutated gene products and tumour-specific splice variants may be useful as targets. In this review we will focus on the features of cancer testis antigens.

Cancer testis antigens as targets for cancer vaccination

The search for tumour-specific antigens revealed a novel class of antigens with an intriguing expression pattern. CT antigens are expressed by a variable proportion, ranging from 10 to 70% of a wide range of different human tumour types. In normal tissues, cancer testis antigens (CTA) and their respective encoding genes, the so-called cancer testis genes (CTG) are not expressed, except for testis. Interestingly, the prototypes of this category, MAGE[2], BAGE[14] and GAGE[15], were initially identified as targets for cytotoxic T cells. Several new members which have been added by SEREX to this category will be discussed in more detail below.

HOM-MEL-40/SSX-2

The HOM-MEL-40 antigen which was detected in a melanoma library is the first cancer testis antigen identified by SEREX. It is encoded by the SSX-2 gene. The members of the SSX genes, SSX1 and SSX2, have been shown to be involved in the t(X;18)(p11.2; q11.2) translocation which is found in the majority of human synovial sarcomas[16]. By this translocation the SSX genes fuse with the SYT gene from chromosome 18 resulting in the hybrid transcript -5' SYT-SSX 3'- which codes for a fusion protein. We observed that the SSX genes are silenced in normal tissues except for testis but are expressed in a wide variety of human tumours. Interestingly, the transcripts expressed in neoplasms other than synovial sarcoma are all derived from nonmutated, nontranslocated genes. Using homology cloning, additional members of the SSX family were identified[17] revealing at least five genes, of which four (SSX-1, 2, 4 and 5) demonstrate a CT-type expression[18,19]. In the meantime we have identified several antigenic peptides derived from SSX gene products (unpublished results).

NY-ESO-1

By applying the SEREX methodology to oesophageal squamous cell carcinoma, Chen et al.[20] identified NY-ESO-1 as a new CTA. NY-ESO-1 mRNA expression is detectable in a variable proportion of a wide array of human cancers, including melanomas, breast cancer, bladder cancer and prostate cancer. A homologous gene, named LAGE-1, was subsequently isolated by a subtractive cloning approach[21]

demonstrating that NY-ESO-1 belongs to a gene family with at least two members. Interestingly, NY-ESO-1 was recently also identified by the CTL cloning approach using melanoma-derived tumour infiltrating lymphocytes[22]. That NY-ESO-1 may be simultaneously an immune target for both antibody and CTL responses in the same patient was demonstrated by studies of Jäger et al.[23]. Stockert et al.[24] observed that IgG antibody responses directed against NY-ESO-1 are present in up to 50% of antigen-expressing patients, indicating that this antigen may also be an important target for CD4+ T lymphocytes.

HOM-TES-14/SCP-1

The expression of CT antigens in tumours and testis prompted our group to modify the original SEREX technique in order to bias for the detection of members of the CT class. For this intention, testis expression libraries were enriched for testis-specific transcripts by subtractive techniques and immunoscreened with allogeneic sera from cancer patients. SEREX screening using such testis-specific surrogate libraries proved to be a successful strategy for the identification of additional CTA[25]. One of the identified new CTAs was shown to be encoded by the gene coding for the synaptonemal complex protein-1 (SCP-1). SCP-1 is known to be selectively expressed during the meiotic prophase of spermatocytes and is involved in the pairing of homologous chromosomes[26], an essential step for the generation of haploid cells in meiosis I. Investigation of a broad spectrum of normal and malignant tissues revealed expression of SCP-1 transcripts and antigen selectively in a variety of neoplastic tissues and tumour cell lines. Immunofluorescence microscopy analysis with specific antiserum showed a cell cycle phase-independent nuclear expression of SCP-1 protein in cancer cells. SCP-1 is hitherto the only CTA with a known function. It is therefore intriguing to speculate which role aberrant expression of a meiotic protein in a somatic cell plays for the genomic instability of cancer cells.

To cope with the rapidly growing number of CTAs, a new nomenclature has been suggested. According to the order of this initial identification the individual genes are designated by enumeration. Thus CT-1 (CT-1.1 – CT-1.13) represents MAGE members, CT-2 BAGE, CT-3 GAGE, CT-4 stands for the SSX-family members, etc. Since individual CTAs are expressed only in a variable proportion of tumours, only the availability of several CTAs could significantly enlarge the proportion of patients eligible for vaccination studies. In this regard it is interesting that members of a given gene family tend to be expressed in a co-regulated fashion, whereas different gene families are preferentially expressed in other sets of tumours[27]. It is therefore reasonable to choose antigens from different CT families to cover as many tumours as possible. Despite the fact that SEREX enlarged the pool of available tumour antigens, the proportion of antigen-negative tumours is still high,

particularly in frequent neoplasms such as colon and prostate cancer. Moreover, immunohistological investigations for MAGE antigens have demonstrated a heterogeneity of antigen expression even in the same tumour specimen[28]. Thus, the combined or sequential use of a whole set of several antigens in a patient would have the potential of reducing or even preventing the in vivo selection of antigen loss tumour cell variants and would also address the problem of a heterogeneous expression of a given antigen in an individual tumour specimen.

Recognition of SEREX antigens by T lymphocytes

As stated above, antitumour immune responses result from a concerted immunological action which involves both cellular and humoral effector mechanisms. Since the isotype switching and the development of high-titred IgG in vivo requires cognate CD4+ T-cell help, SEREX can be instrumentalized to analyse the CD4+ T-cell repertoire against tumour antigens. With regard to CD8+ T lymphocytes that are recognizing SEREX antigens, it is noteworthy that MAGE-1, MAGE-4a and tyrosinase originally described as CTL targets have also shown up during the SEREX immunoscreening of several tumours, suggesting that at least some of the serologically identified antigens may bear epitopes that are recognized by CTL. Moreover, CTL responses have been demonstrated by two independent groups for the SEREX-defined NY-ESO-1 antigen and resulted in the identification of three HLA-A201 and three HLA-A31 restricted epitopes. Interestingly, antigenic peptides for two of the three HLA-A31 restricted epitopes are encoded by an alternative open reading frame indicating that CTLs may respond to two different gene products translated from alternative reading frames of the same gene.

Identification of T-cell epitopes of SEREX antigens

The search for T-lymphocyte-recognized epitopes of defined molecules is an important new field of molecular tumour immunology and has been titled 'reverse T-cell immunology' by Thierry Boon. Because of the diversity of peptides presented by the highly polymorphic HLA alleles this objective means an enormous challenge for each individual antigen. Our group is also addressing this question in detail for members of the CTA class identified by SEREX. Several strategies have been created and used in recent years for this purpose.

The antigenic peptide approach

Using different algorithms, the sequences of serologically identified antigens are scanned for peptides containing binding motifs for MHC I or MHC II alleles[29,30]. Due to the polymorphism of HLA we focus on well characterized and frequent

MHC alleles. The predicted peptides are synthesized and tested for binding to the respective MHC molecules. Peptides with affinity to MHC molecules are loaded onto dendritic cells or other professional antigen presenters and used to stimulate autologous T lymphocytes. T cells expanded by repeated stimulation are tested for HLA-restricted reactivity to antigen-positive target cells. Using this approach we have identified several HLA-A201-presented antigenic peptides for different SEREX antigens.

The whole-protein approach

Another strategy is based on the utilization of the full-length antigen which is either expressed in professional antigen-presenting cells by polynucleotide transfection or is fed as recombinant protein. Successful transfection of polynucleotides in dendritic cells has been described using in vitro translated RNA[31] or recombinant viral delivery systems, such as vaccinia or adenovirus[32]. Dendritic cells presenting antigenic peptides after processing of endogenously expressed or exogenously loaded antigens are used for repeated stimulation of autologous T lymphocytes. Similar to the peptide approach expanded T lymphocytes are tested for antigen-specific reactivity and MHC restriction.

The pre-existing CTL approach

A multitude of CTL clones with tumour cell specific reactivity has been established worldwide and in many cases the identification of the respective target antigens has resisted scrutiny. Using COS cells co-transfected with cDNA coding for serologically identified antigens together with the restriction element, the reactivity of these T cells against SEREX antigens can be easily tested.

Despite the fact that reverse T-cell immunology is a new terrain in tumour immunology the elaboration of technical advances provides rapid progress in this field. The analysis of peptides eluted from MHC molecules by mass spectroscopy is becoming more and more sensitive and will assist in the direct identification of naturally processed peptides derived from particular antigens. Together with the typing of the immunogenic genome provided by SEREX this will shape an ever more complete picture of the repertoire of cancer-associated antigenic peptides. The knowledge derived from these studies will form the basis for a rational immunotherapy, the success and failure of which could be analysed at the molecular level.

Conclusions and prospects for the future

The multitude of tumour-specific antigens identified by the SEREX technique has revealed that the immune recognition of human tumours by the autologous host's

immune system is not impaired and opens the perspective for depicting an antigenic profile for each individual tumour. With the identification and molecular definition of multiple antigens expressed by a given tumour, which elicit an immune response in the autologous cancer patient, it has now become evident that the recognition of tumour antigens is not the limiting step in immune responses against tumours. Rather, it is more likely that it is the effector arm of the immune system that contributes to the failure of the cancer patient's immune system to prevent or control cancer. The availability of molecular defined genes, which are specifically expressed or overexpressed in many (and possibly all) human cancers, now provides a tool for the redirection or upregulation of the effector arm of the immune response towards an efficient cytotoxic response against malignant cells using various cancer vaccine strategies. The study and long-term follow-up of large numbers of patients will help to determine the diagnostic and prognostic relevance of tumour-specific antibodies in patients' sera and of antigen expression in tumours, as well as CTL responses. The abundance of human tumour antigens will enable us to proceed with the development of polyvalent vaccines for a wide spectrum of human cancers using pure preparations of molecular defined antigens or antigenic peptide fragments.

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